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(54) Title: CYTOPLASMIC DIPEPTIDYLPEPTIDASE IV FROM HUMAN T–CELLS

(57) Abstract

A purified enzyme characterized as follows: (a) it is present in the cytoplasm of Jurkat cells; (b) it exhibits specific post–prolyl dipeptidase enzymatic activity; (c) it is structurally distinct from CD26; (d) its dipeptidase activity is reduced more than ten–fold when acting at a pH of 5.5, compared to a pH of 6.8; (e) it has an apparent molecular weight of about 60kD on SDS–PAGE; (f) it contains the amino acid sequence NAFTVLAMMDYPY [GT148], and DLFLOGAYDTVR [GT103]; (g) it contains the amino acid sequence LDHFNFER [GT85], and DVTADFEGOSPK [GT69]; (h) it occurs naturally in T–cells of healthy humans; (i) it naturally protects human T–cells in which it occurs from apoptosis; (j) it is multimeric; and (k) it has an isoelectric point of 4.5–5.5, as measured by ion–exchange.

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CYTOPLASMIC DIPEPTIDYLPEPTIDASE IV FROM HUMAN T-CELLS

Background of the Invention

This invention relates to T-cell enzymes, and to apoptosis, or programmed cell death (PCD).

Apoptosis shapes a number of diverse biological processes, including development and homeostasis of the immune system. Apoptosis is the physiologic form of cell death that regulates development and homeostasis. Apoptosis occurs in response to diverse stimuli which fall into two categories: activation induced cell death following specific stimulation, and death by neglect after withdrawal of life promoting stimulation. While these two types of PCD take place under very different circumstances, both depend on the activation of caspases, a family of cysteine proteases which are present in the cytoplasm of cells as inactive proenzymes.

Apoptotic stimuli lead to the activation of certain caspases by specific proteolytic cleavage, enabling them to activate other caspases through a proteolytic cascade, which eventually leads to cell death. Studies of activation induced cell death through the Fas/TNF receptors have implicated the death effector domain containing protease FLICE (caspase 8) in the initiation of the caspase cascade. However, while most cells contain all the components of the apoptotic machinery and are susceptible to PCD by neglect, e.g., factor/serum withdrawal or loss of cell-cell contact, no regulator of the caspase cascade induced under these conditions has thus far been identified.

Summary of the Invention

We have discovered a human T-cell cytoplasmic post-prolyl dipeptidase which has similarities to, but is distinct from, the membrane-bound

T-cell serine protease CD26. This new enzyme, which we have termed DPIVb, is provided according to the invention in purified form.

We have discovered that DPIVb is naturally present in T-cells in healthy individuals, and is involved in the protection of those T-cells from apoptosis. A cell-death-related property of DPIVb was discovered in the context of HIV infection. Our observations led us to hypothesize that the resistance to full activation observed in T-cells of HIV-infected individuals involves a block of DPIVb activity, which prevents differentiation of T-cells of HIV-infected individuals into effector cells, eventually leading to T-cell death. The new cytoplasmic serine protease DPIVb exhibits activity which prevents, rather than promotes, apoptosis in resting T-cells. This non-proteasomal enzyme thus is essential for the survival of quiescent T-cells, preventing cell death by blocking the catalytic activation of caspases. The identification of this life- promoting serine protease reflects the emerging importance of non-proteasomal enzymes as key regulators of cell survival. The dipeptidase DPIVb activity of the invention is present in the cytoplasm of a number of types of normal, resting human T-cells, e.g., CD4 cells and Jurkat cells.

Accordingly, the invention features a novel, purified enzyme characterized as follows:

a. it is present in the cytoplasm of Jurkat cells;

b. it exhibits specific post-prolyl dipeptidase enzymatic activity;

c. it is structurally distinct from CD26;

d. its dipeptidase activity is reduced more than 50% when acting at a pH of 5.5, compared to a pH of 6.8;

e. it has an apparent molecular weight of about 60 kD on SDS-PAGE;

f. it contains the amino acid sequence NAFTVLAMMDYPY

[GT148] and DLFLOGAYDTVR [GT103];

g. it contains the amino acid sequence LDHFNFER [GT85], and DVTADFEGOSPK [GT69];

h. it occurs naturally in T-cells of healthy humans;

i. it naturally protects human T-cells in which it occurs from apoptosis;

j. it is multimeric; and

k. it has an isoelectric point of 4.5-5.5, as measured by ioin-exchange.

The enzyme of the invention can be used therapeutically to treat a patient having a medical disorder requiring protection of the patient's T-cells from cell death, by administering the enzyme, in a therapeutic formulation, to the patient in a T-cell protective amount.

The purified DPIVb of the invention can also be used to screen compounds for the ability to inhibit the novel enzyme; the screening is carried out using standard enzyme inhibition measuring techniques, involving contacting the test compound with DPIVb and measuring DPIVb activity (against a control absent the test compound) to determine whether the test compound inhibits the enzyme. Compounds which are inhibitory are candidates for use in therapy in which death of certain cells is therapeutically desirable. For example, in some T-cell neoplastic diseases, e.g., certain leukemias and lymphomas, it may be desirable to de-protect the cancerous T-cells from endogenous DPIVb, by inhibiting the enzyme and thus promoting the death of these cells.

The purified DPIVb of the invention can also be used to make antibodies (polyclonal, monoclonal, or recombinant) using conventional methods, involving immunization of, e.g., rabbits, mice, or human volunteers.

The antibodies can be used in standard ELISA assays to measure DPIVb levels in patients being tested for diseases which potentially involve increased or decreased DPIVb levels; for example, HIV patients, who have lost DPIVb-containing T-cells, will exhibit decreased DPIVb levels, with the DPIVb concentration being diagnostic of the stage of the disease. Generally, because DPIVb is a cytoplasmic enzyme, the assay is carried out on peripheral blood lymphocyte samples which have first been treated to lyse T-cells to release the enzyme.

By "purified" enzyme is meant DPIVb that has been separated from components which naturally accompany it. The enzyme is substantially pure when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, pure DPIVb by weight. Purity can be measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

Other features and advantages of the invention will be apparent from the following detailed description thereof, and from the claims.

Detailed Description

The work leading up to the discovery of the new cytoplasmic enzyme of the invention began with the question of whether, in resting cells, the apoptotic machinery may be kept inactive by the basal activity of cellular enzymes. We screened various enzymatic inhibitors for their ability to trigger PCD in overnight cultures of freshly isolated peripheral blood mononuclear cells (PBMC).

We observed a striking increase in the number of dead cells in cultures containing the L-isomer of Val-boroPro (VbP), an inhibitor of dipeptidyl peptidase IV (DPPIV), compared to cultures containing media alone or the inactive D-isomer of the inhibitor, d-Val-d-boroPro--a toxicity control. Dead cells were apparent as early as 4 h after the addition of the L-isomer of VbP, with maximal death occurring within 24 h (about 70%). When subpopulations of PBMC were tested for susceptibility to VbP- induced death, we observed that $CD19^+$ B cells and $CD11b^+$ monocytes were resistant, while purified T-cells ($CD4^+/CD8^+$) showed greater sensitivity than whole PBMC. These results imply that the molecular target of VbP plays a role in T-cell survival.

The appearance of phosphatidylserine (PS) on the outer leaflet of the plasma membrane is a caspase dependent characteristic of apoptotic cells. Using the PS binding protein FITC-annexin V, we detected a time dependent increase in the amount of PS on the surface of cells treated with VbP, establishing that VbP induces apoptosis. We confirmed that VbP induces PCD through the activation of the caspase cascade by showing that the general caspase inhibitor Z-VADfmk blocks VbP-mediated T-cell death.

We next determined if VbP- induced death proceeds through Fas, the well defined and prototypic activation induced death pathway of T-cells, or the proteasome pathway. Although these pathways affect activated cells, and PBMC are predominantly resting cells with only basal metabolic activity, exposure to small molecule drugs like VbP may induce the expression of Fas ligand (FasL) or have other unanticipated effects that activate these pathways. However, the anti-Fas mAb M3, which prevents PCD by blocking Fas-FasL interactions, had no effect on VbP mediated death of T-cells, indicating that VbP does not induce the expression of FasL. Lactacystin, an inhibitor of

proteasome activity, induces cell death in cycling cells through the disregulation of proteins necessary for cell cycle progression, and does not induce PCD in resting PBMC. However, after activation, PBMC are susceptible to lactacystin induced death. Surprisingly, activated PBMC are resistant to VbP induced PCD, but regain sensitivity if maintained in culture until the cells return to G_0. These findings confirm that VbP- mediated PCD in resting cells proceeds through the caspase cascade and is independent of Fas-FasL interactions or a proteasome-mediated cell cycle block.

VbP was designed to inhibit DPIV, a catalytic activity attributed to the surface memory T-cell antigen CD26. We discovered that CD26 is not the only target of VbP; namely, we observed that $CD26^+$ and $CD26^-$ T-cell subpopulations in their resting state are equally sensitive to PCD induction by VbP, indicating that a protein(s) distinct from CD26 mediates VbP induced death. Since VbP is a highly potent inhibitor of DPIV, we reasoned that the PCD inducing target of VbP is an enzyme with substrate specificity similar to that of DPIV. We began to characterize this target by assaying cleavage of the DPIV substrate AlaProAFC.

To biochemically and pharmacologically identify this new target of VbP, we prepared membrane and cytosolic fractions from freshly isolated PBMC. The cytosolic preparation (110,000 g supernatant) contained a pH dependent AlaProAFC cleaving enzymatic activity. This activity is not the result of DPIV action, because the integral membrane protein CD26 fractionates with the membranes (110,000 g pellet). Optimal AlaProAFC cleavage occurred at pH 7.5, excluding lysosomal proteases as the source of this activity. The enzymatic activity was inhibited by VbP and partially inhibited by high concentrations of benzamidine and the serine or cysteine protease active site titrants PMSF, TLCK, NEM and IAA, which most likely

act as nonspecific alkylating agents at these high concentrations. Furthermore, peptidyl boronic acids, like VbP, are effective inhibitors of serine proteases, but do not inhibit cysteine proteases, indicating that the cytosolic enzyme responsible for AlaProAFC cleavage belongs to the serine class of proteases.

Because of the similar substrate specificities of the new cytoplasmic peptidase DPIVb and the known enzyme DPIV, we examined the inhibitory potential of several known DPIV peptidase inhibitors on DPIVb. Inhibition was achieved with similar concentrations of VbP and NO_2-Z-Lys-thiazolidide (Lys-thyazolidide), the most potent inhibitors of this activity. The related compound NO_2-Z-Lys-piperidide (Lys-piperidide) was an effective inhibitor at higher concentrations than Lys-thiazolidide, and the fluoroolefin containing (Z)-Ala-Ψ[CF=C]-Pro-NHO-Bz(4NO_2)(L-125) did not inhibit DPIVb. The inhibitory potential of these compounds confirms that DPIVb is different from DPIV, as DPIV is inhibited by VbP, L-125, and Lys-thiazolidide, but not by Lys-piperidide. Furthermore, inhibition of AlaProAFC cleaving activity by these compounds in cytoplasmic preparations completely correlated with PCD induction in PBMC cultures.

Our functional and pharmacological data strongly suggest that DPIVb is a regulator of the caspase cascade in quiescent T-cells, supporting a novel model for the regulation of this pathway. In resting T-cells the caspase cascade is kept inactive through the action of DPIVb; inhibition of this peptidase activity allows activation of the caspase cascade, leading to cell death. The implication of this model, that resting T cells are poised to die, is consistent with the notion that PCD is a constitutive program which must be suppressed. Resting T-cells seem to be the sole target of VbP induced death in PBMC, despite the presence of similar peptidase activities in the cytoplasm of activated T-cells and other blood cells. Interestingly, we observed a 9 fold

reduction of AlaProAFC specific cleaving activity in cytoplasmic extracts from PHA activated T-cell blasts compared to that of quiescent PBMC, suggesting that this activity is replaced by other inducible proteolytic activities upon cellular activation. Thus, we anticipate that this proline peptidase is the first identified member of a family of proteolytic activities which regulate the caspase cascade in quiescent cells.

Methods

Human PBMC were isolated from the blood of healthy doners by centrifugation on a Ficoll-Hypaque gradient using standard techniques. T-cells and monocytes were isolated by sorting on a Becton Dickinson FACStar plus flow cytometer using biotinyl-anti-CD4 plus biotinyl-anti-CD8 (Leu2a plus Leu2b, Becton Dickinson) or anti-CD11b (44-biotin, Sigma), and phycoerythrin streptavidin, $CD26^+$ T cells were isolated by sorting with the anti-CD26 mAb 1F7 (C. Morimoto, Dana-Farber Cancer Inst.). B cells were isolated by selection with biotinyl-anti-CD19 mAb (D. Thorley Lawson, Tufts Univ.) And MACS microbeads (Miltenyl Biotec). Sorted cell populations were >90% pure. Cells were washed several times in PBS and resuspended in serum free AIM V medium (Gibco), and plated at 1-2 x 10^5 cells/well in 96 well flat bottom plates. Enzyme inhibitors and mAb were added and the cells incubated for 18-24 h in 5% CO_2 at 37°C. Cellular death was quantitated by staining the cells with 20 μg/ml 7-amino actinomycin D (Sigma), hypotonic propidium iodide (Sigma), or FITC-annexin V (Southern Biotech Associates). Activated cells (PHA Blasts) were stimulated for 3 days with 5μg/ml phytohemagglutinin (PHA, Sigma). Long Term Cultured cells were cultured with PHA for 3 days, washed several times and cultured an additional 32 days without stimulating agents. Additional reagents: anti-FAS mAb M3 (D. Lynch, Immunex); Z-VADfmk (Enzyme System Products); lactacystin (E.J. Corey, Harvard Univ.);

VbP, Lys-piperidide, Lys-thiazolidide (R. Snbow and A. Kabcenell, Boehringer Ingelheim Pharmaceuticals), L-125 (J. Welch, State Univ. of N.Y. at Albany).

Enzyme Characterization

Human PBMC (~430 x 10^6 cells) were isolated from 450 ml whole blood. Cells were resuspended in 7 ml ice cold lysis buffer (0.02 M Tris pH 7.8, 4μg/ml aprotinin, 8μg/ml leupeptin, 8μg/ml antipain) and held on ice 10 min before homogenization by 10 strokes of a Dounce homogenizer. EDTA was added to a final concentration of 5mM, and the homogenate centrifuged at 1000 g for 10 min. at 4°C. The resulting supernatant was centrifuged at 45,000 g for 20 min. At 4°C. The 45,000 g supernatant was centrifuged at 110,000 g for 1 hour at 4°C, the 110,000 g supernatant was used as soluble cytoplasmic extract. The 45K and 110K pellets were combined, resuspended in 10 ml lysis buffer, and centrifuged at 110,000 g for 20 min. at 4°C. The resulting pellets were combined and resuspended in lysis buffer/1% Triton X-100 and used as the membrane preparation. Peptidase activity was measured by monitoring the accumulation of the fluorescent product 7-amino-4-trifluoromethylcoumarin (AFC) liberated from the substrate AlaProAFC (Enzyme Systems Products) for 1 min., using a Perkin-Elmer fluorescence spectophotometer (excitation 400 nm, emission 505 nm). The optimal pH for AlaProAFC cleavage was determined using: 0.05 M acetate pH 4.5, 5.0; 0.2 M piperazine pH 5.5, 0.05 M NaPhosphate pH 6.0, 6.5, 7.0; 0.05 M HEPES pH 7.5, 8.0, 0.02 M TRIS pH 8.5, 9.0; 0.05 M HEPES pH 7.5 was used for all subsequent enzymatic assays. The general protease inhibitors: PMSF, benzamidine, TLCK, aptrotinin, leupeptin, IAA and NEM were purchased from Sigma. All reported catalytic rates are the average of 3 independant determinations.

We detected an enzymatic activity in the soluble fraction of CD26-Jurkat cells which cleaves the substrate AlaProAFC. The activity is inhibited

by the DPIV inhibitors XaaboroPro (described in Bachovchin et al. U.S. Patent No. 5,462,928, hereby incorporated by reference). However, the activity is distinct from DPIV based upon differential sensitivity to other inhibitors (thiazolidide, piperidide, PMSF). The activity is greatly reduced (more than 50%) at pH 5.5.

DPIVb Purification

We purified DPIVb from Jurkat cytoplasm. This scheme produces a 1000-fold purification of DPIVb with a 27% yield:

l. Jurkat cytoplasm (110,000 g supernatant)

m. Acid soluble fraction (0.05 M acetate pH 4.5)

n. Cation Exchange (SP)

o. Gel filtration (Superdex 12)

In more detail, the above-outlined purification scheme is carried out as follows. First, Jurkat cells (10^6-10^{11} cells) are grown and a cell pellet is obtained by centrifugation. The cell pellet is stored in frozen condition.

The frozen pellet is thawed by the addition of ice cold lysis buffer, in the amount of approximately 1 ml per 10^8 cells. The liquified material is homogenized with ten strokes of a Dounce homogenizer, and then clarified by centrifugation at 1500 g. The supernatant is removed (and reserved), and the 1500 g pellet is resuspended in lysis buffer and homogenized with ten strokes of a Dounce homogenizer. Clarification is again carried out by centrifugation at 1500 g at 4°C.

The 1500 g supernatants are then combined, and EDTA is added to 5 mM. The resultant liquid is centrifuged at 75,000 g at 4°C for twenty minutes, and the supernatant is then removed and centrifuged at 110,000 g at 4°C, for 60 minutes. The resultant supernatant, containing the cytosolic extract, is the

DPIVb activity-containing fraction on which the subsequent purification steps are carried out.

This procedure does not yield a homogenous preparation, and attempts at further purification (ion exchange, affinity chromatography) resulted in the loss of nearly all protein in the sample, and recovery of very little activity.

To identify the component of this purified preparation responsible for XaaPro-cleaving activity, we established that diisopropylfluorophosphate (DFP) inhibits the activity in the SP purified fraction. An aliquot of the most pure preparation was then labeled with 3H-DFP and separated by SDS-PAGE. Radiofluorography of the dried gel revealed a specifically labeled protein of ~60 kD. The corresponding band from a Coomassie stained gel contained purified DPIVb.

DPIVb Peptide Sequences

Sequences of four peptides of a DPIVb tryptic digest are given in Figs. 1 and 2. Peptide GT148 (Fig. 1) is 13 residues long and has some homology to residues 281-293 of human prolylcarboxypeptidase (PCP). Peptide GT85 (Fig. 2) is 8 amino acids long and is identical to residues 17-24 of swine dipeptidyl peptidase II (DPPII).

Use in Screening

Purified DPIVb of the invention can be used to screen compounds which inhibit the enzyme, thereby hastening T-cell death. Compounds that kill T-cells can be used as immunomodulating drugs for the treatment, e.g., of allograft rejection, graft-versus-host disease, and auto-immune diseases such as rheumatoid arthritis.

Screening is carried out using a reporter substrate which contains proline in the penultimate position; any of a number of substrates meeting this

requirement can be used. One suitable assay is a fluorescent cleavage assay using the substrate AlaProAFC. Alternatively, a colorimetric assay can be carried out using as a substrate Gly-Pro-pNA. The choice of terminal amino acid is not critical, provided that the substrate contain a free terminal amino group.

A fluorescence assay employs a fluorescence spectrometer for excitation at 400 nm and emission at 505 nm. The spectrometer is calibrated for fluorescence intensity of 0.000=10 mM HEPES, pH 7.4; and fluorescence intensity of 1.000=10 Mm HEPES, 1μM AFC.

To carry out the assay, between 10 and 100 μl of purified DPIVb enzyme, is diluted to 1 ml with 10 mM HEPES, pH 7.4, containing 10 mM Ala-Pro-AFC. At least one extract/substrate sample is run without test compound, to provide a standard for comparison with the test sample.

In the test samples, multiple samples are run containing varying concentrations, down to 10^{-8}M, of the test compound. The sample (with or without test compound) is placed in a cuvette, and inserted into a fluorescent spectrometer. Enzymatic activity is measured as the accumulation of fluorescence intensity (i.e., substrate cleavage product) over time (1 min.). A compound is identified as an inhibitor if fluorescence is decreased as a result of the presence of the inhibiting compound.

Once a compound has been identified as an inhibitor, further assays are carried out to determine whether the compound is capable of moving across the T-cell membrane into the cytoplasm; this is an assay which can be carried out using well-known techniques.

The candidate compounds screened using DPIVb should be organic compounds which have a free amino group at the amino terminus; a proline or proline analog at the penultimate position; and an enzyme binding site which

mimics the post-prolyl cleavage site of DPIVb.

A number of known classes of compounds can be screened and used according to the invention. Once such class are CD26 (i.e., DPIV) inhibitors, including those described in Bachovchin et al. U.S. Patent No. 4,935,493, *id.*

Sources of DPIVb

DPIVb can be obtained by purification from human lymphocytes, as described herein. Alternatively, the enzyme can be produced recombinantly using known techniques, e.g., expression of the DPIVb coding sequence in mammalian cells such as Chinese hamster ovary cells.

Use as Therapeutic

Because the purified DPIVb enzyme of the invention is protective of death in normal resting human T-cells, it can be administered therapeutically to patients in need of immune system enhancement, and in particular protection of clinically important T-cell subsets such as $CD4^+$ cells. Such patients include AIDS patients whose $CD4^+$ cell counts have fallen as a result of their illness; and cancer patients who have suppressed immune function as a result of their disease, chemotherapy, and/or radiation therapy.

Administration

DPIVb enzyme may be administered by any appropriate route. For example, administration may be parenteral, intravenous, intra-arterial, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, by suppositories, or oral administration.

Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found, for example, in "Remington's Pharmaceutical Sciences." Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel. The concentration of DPIVb in the formulation will vary depending upon a number of factors, including the dosage of the drug to be administered, and the route of administration.

The formulations can be administered to human patients in therapeutically effective amounts (e.g., amounts which eliminate or reduce the pathological condition) to provide therapy for the disorders described above. Typical dose ranges are from about 0.1 μg/kg to about 1 g/kg of body weight per day. The preferred dosage of drug to be administered is likely to depend on such variables as the type and extent of the disorder, the overall health status of the particular patient, the formulation of the compound excipients, and its route of administration.

Antibodies Directed against DPIVb

The purified DPIVb of the invention, or fragments thereof, can be used

to generate polyclonal or monoclonal antibodies specific for DPIVb, using conventional techniques. Such antibodies can be used in any of the many known conventional immunoassay formats to measure DPIVb levels in biological samples, e.g., samples of peripheral blood lymphocytes. A decreased level of DPIVb as determined in such assays is indicative of immune dysfunction in the patient from whom the sample was obtained.

Other Embodiments

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure come within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth, and follows in the scope of the appended claims.

Other embodiments are within the claims.

What is claimed is:

Claims

1. A purified enzyme characterized as follows:

a. it is present in the cytoplasm of Jurkat cells;

b. it exhibits specific post-prolyl dipeptidase enzymatic activity;

c. it is structurally distinct from CD26;

d. its dipeptidase activity is reduced more than ten-fold when acting at a pH of 5.5, compared to a pH of 6.8;

e. it has an apparent molecular weight of about 60 kD on SDS-PAGE;

f. it contains the amino acid sequence NAFTVLAMMDYPY [GT148],
and DLFLOGAYDTVR [GT103];

g. it contains the amino acid sequence LDHFNFER [GT85], and DVTADFEGOSPK [GT69];

h. it occurs naturally in T-cells of healthy humans;

i. it naturally protects human T-cells in which it occurs from apoptosis;

j. it is multimeric; and

k. it has an isoelectric point of 4.5-5.5, as measured by ion-exchange.

2. A method of protecting T-cells from cell death, said method comprising contacting said T-cells with a T-cell protective amount of a therapeutic composition comprising purified DPIVb.

3. A therapeutic composition comprising purified DPIVb enzyme.

4. A method of determining whether a test compound is capable of inhibiting DPIVb, said method comprising

a) contacting said compound with DPIVb and,

b) measuring DPIVb activity to determine whether said test compound inhibits DPIVb.

5. An antibody specific for DPIVb.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/20968

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) : A61K 38/48; C12N 9/48
US CL : 424/94.64; 435/212
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.64; 435/212

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, JPOABS, EPOABS, REG, INDEX BIOSCIENCE
search terms: dipeptidylpeptidase IV (and variants), CD26, cytoplasm?, cytosol?, soluble, jurkat, t-cell, human

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KABCENELL et al. 'Mechanism of Action of Peptide Inhibitors of CD26 in T Cell Activation: Evidence Against a Direct Effect on Dipeptidyl Peptidase IV.' Keystone Meetings, 20-26 March 1996.	1
A	TAN et al. Sequencing and Cloning of Human Prolylcarboxypeptidase (Angiotensinase C): Similarity to Both Serine Carboxypeptidase and Prolylendopeptidase Families. J. Biol. Chem. 05 August 1993, Vol. 268, No. 22, pages 16631-16638.	1-3

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
15 DECEMBER 1998

Date of mailing of the international search report
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/20968

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DUKE-COHAN et al. A Novel Form of Dipeptidylpeptidase IV Found In Human Serum: Isolation, Characterization, and Comparison with T Lymphocyte Membrane Dipeptidylpeptidase IV (CD26). J. Biol. Chem. 09 June 1995, Vol. 270, No. 23, pages 14107-14114.	1-3
A	LIU et al. Progesterone-Induced Secretion of Dipeptidyl Peptidase-IV (Cluster Differentiation Antigen-26) by the Uterine Endometrium of the Ewe and Cow That Costimulates Lymphocyte Proliferation. Endocrinology. February 1995, Vol. 136, No. 2, pages 779-787.	1-3
A	DUKE-COHAN et al. Serum High Molecular Weight Dipeptidyl Peptidase IV (CD26) Is Similar to a Novel Antigen DPPT-L Released from Activated T Cells. J. Immunol., 01 March 1996, Vol. 156, No. 5, pages 1714-1721.	1-3
A	KÄHNE et al. Alterations in Structure and Cellular Localization of Molecular Forms of DP IV/CD26 During T Cell Activation. Cellular Immunology. 25 May 1996, Vol. 170, No. 1, pages 63-70.	1-3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/20968

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1 (in part)
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Please See Extra Sheet.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-3

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/20968

BOX I. OBSERVATIONS WHERE CLAIMS WERE FOUND UNSEARCHABLE
2. Where no meaningful search could be carried out, specifically:

Claim 1 is at least partially directed to sequences of tryptic peptides contained within the complete sequence of the enzyme. The sequences were unsearchable because a) there was no compliance with the Sequence Rules, and b) two of the four peptide sequences contain the one letter code "O" which does not correspond to any of the twenty amino acids known to naturally occur in proteins. As a consequence, the claims were searched in part insofar as they could be searched on the basis of other claimed properties of the enzyme.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-3, drawn to DP-IVb *per se* and pharmaceutical composition thereof, and method of treatment with DP-IVb.
Group II, claim 4, drawn to a method of screening for inhibitors of DP-IVb.
Group III, claim 5, drawn to antibodies that recognize DP-IVb.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The method of screening is distinct from the method of treatment, sharing no steps in common. Inhibitors resulting from a screening would be used in a different therapy from the DP-IVb itself. Antibodies which bind to DP-IVb are clearly distinctly different products from DP-IVb itself. Substrates, inhibitors, regulators and salts also bind to DP-IVb.